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AN ATTEMPT AT A SUBJECTIVE AND OBJECTIVE CLASSIFICATION OF THE ACIDOPHILIC GRANULOCYTES OF SOME MARINE FISHES[1]

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The study of the acidophils in the blood smears of fishes has been very limited. Drzwena (1911) in the most extensive study of the morphology and tinctorial reactions of the acidophils of the blood smears of fishes, fixed by heat and stained with acid or basic dyes, concluded that the acidophils or eosinophils are those cells in which the granules stain only with the acid dye. She distinguished a tinctorial difference from light orange (indicative of faint eosinophilia) to deep wine red (indicative of strong eosinophilia). The cells could be of any size or shape, the nucleus of any shape, the granules of any size or shape, and there could or could not be a basophilic matrix. She studied the eosinophils of the Chondrichthyes and Osteichthyes and found great variation in the sizes and shapes of the granules and their tinctorial reactions even in closely related species. In this report I am presenting evidence for the classification of such cells not only by subjective description, but by their transmission of monochromatic light.

The basic assumption upon which this report is based, is that if monochromatic light is passed through a film or filter with the same color as the wave length of the light source, the light passed through will have the same value except for some reflection or absorption depending upon the material of the filter (Weiskopf, 1968).

From a study of the objective types of acidophils it was hoped that the objective measurements of the degrees of transmission of monochromatic red light could be made directly on the stained cells. However, it was found that in order to get sufficient magnification to do this, the heat transmitted at the same time was so great that the dyes faded rapidly. Consequently, it was decided to make an objective classification of these subjectively classified acidophils by measuring the amount of monochromatic red light transmitted through photomicrographs of acidophils which had been taken at oil immersion magnification (1250 ×, reduced for photography by $\frac{1}{3}$ by the Leitz adapter) on color film, which in turn was magnified by 41 ×, so the total magnification of the cytoplasm of the acidophils at which the transmission was measured was 16,400 ×.

The objective measurements of the acidophilic material of the acidophils studied by this method when analyzed statistically showed a variety of differences in transmission by acidophils with similar subjective characteristics. It it suggested that these differences, which are based upon transmission by human eosinophils as a control, may be used as an auxiliary tool in taxonomic classification and in experimental investigation. It is hoped that some method based upon this may be devised which is similar to the physical methods used by Stein, Lipkin, and Shapiro (1969) to facilitate optical measurements in biology.

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Methods

The methods of study are simple, but the need for more accurate preparations of the materials for the present observations were not realized until after the blood smears had been stained, and photographs made at different times, on different films. The absolute identity of preparation could not be done, because of variations in the amount of plasma clinging to the cells, the difference in density and probably in chemical content of the plasma, the degree of flattening of the cells in making the smears under varied environmental conditions by different people, and the possible difference in the chemical characteristics of the Wright's stain over the period of several years when the smears were collected and stained. An attempt was made to control such things as could be controlled. The air-dried smears were exposed to 10 drops of Wright's stain for one minute, and a similar amount of distilled water buffered to pH 6.8 was mixed with the stain and allowed to stand for two minutes; the water was rinsed off, the stained smear was dried rapidly in air and stored. This is a standard method for the preparation for study of the blood cells of man, but as pointed out above there were difficulties in staining adsorption because of the plasma. Apparently this did not affect the leucocytes, but erythrocytes often tended to be more blue than red.

The photomicrographs were made on the same Leitz Aristophot under oil immersion at a magnification of 1250 ×, reduced by the $\frac{1}{3}$ adapter. The source of light for photography was 1.2 foot candles with daylight (80C) filter, and the exposure time was $\frac{1}{5}$ second on Ektachrome F (speed 16), and $\frac{1}{10}$ of sec with daylight (85) filter on Ektachrome X (speed 64).

Since the observations on the blood cells of the fishes were part of the larger project on the morphology of the blood cells of the vertebrates, an attempt was made to photograph sample cells from each of the fishes. After preliminary study in which counts and descriptions of the acidophilic granulocytes were made from identifications given in the literature by Drzwena (1913) and other investigators and reviewed by Jordan (1938), Kindred (1961), and more recently by Saunders (1966), the photomicrographs were made for this projected atlas. It is from these photomicrographs that the following contrasts between subjective characteristics and objective measurements of transmission of light by the acidophilic materials have been made.

First, the subjective classification has been made on the basis of the presence of acidophilic material alone in the cytoplasm (type A); acidophilic materials and clear spaces (type B); acidophilic materials and basophilic matrix (type C); and acidophilic materials, clear spaces and basophilic matrix (type D). Next, the objective characteristics, the transmission of monochromatic red light (Filter 29W)/1.0 mm^2) by the largest possible area of the cytoplasm was measured as delineated by a masked strip placed over the photomicrograph of the cell as it was viewed on the microscopic stage at a magnification of 41 ×. Since the photomicrograph had been made at a magnification of 400 ×, the total magnification of the viewed cell was 16,400 ×. Specially prepared diagrams of the areas viewed permitted rapid recording of the photometric measurement of the localized transmission by placing the scanner over the ocular. Since the amount of light passing through the photomicrograph depended upon the area delineated and on the relative amounts of acidophilic material, clear areas, and basophilic matrix, the percentage of each of these areas was measured on projected photomicrographs at a magnification of 5600 ×. The difference between the amount of light transmitted by the photographic film and layer of plasma on the same photomicrograph in an area equal to the extent to that of the cytoplasm measured, and that by the photographic film, plasma and cytoplasm of the cell, gave the actual measurement of transmission by the area of the cytoplasm alone. The role of the different tinctorial reactive areas in the total measurement was then calculated from the ratio of transmission by acidophilic material, clear areas and basophilic matrix to each other. These fundamental data were obtained by measurement of the transmission of the red light from the light source (0.83 foot candles/1.0 mm^2), that transmitted by clear film for the clear area (1.2 ft-c/1.0 mm^2), and for the basophilic matrix (0.22 ft-c/1.0 mm^2). From these data the amount of red light transmitted for each tinctorial area of the cell was calculated and entered in a master table from which only the transmission by the acidophilic area is entered in Figure 1.

Finally, for statistical analysis, the mean transmission of red monochromatic light by the acidophilic elements of eight photomicrographs of human eosinophils, stained with Wright's stain by the same method given above (0.12 ft-c/1.0 mm^2) was used as a control. The X2 deviations from this control by the measurements of transmission by the fish acidophils were used to classify the acidophils objectively (Fig. 1) (Fisher, 1934; Croxton, 1953).

Since the photomicrographs had been taken on either Ektachrome F (speed 16) or Ektachrome X (speed 64), allowing for difference in speed as stated above, the transmission by the photomicrographs of the cells on the two types of film were tested for bias. These calculations indicated that there was no significant bias in the tinctorial reactions of the acidophilic contents, and such deviations as occurred seemed to be dependent on the adsorption of the dye by the acidophilic granules of the cells which had been photographed rather than on the reactions of the film used.

These data and the data on the details of the measurements of the sizes of the cells, areas and transmission by all parts of the cells, calculations for correlations, size, weighting of the samples, and other such basic data which seemed too cumbersome and nonessential for publication in such a pilot study are filed if wanted for further reference. The chief results of pertinence are presented in Figure 1 which depicts graphically the transmissions in their relations to the species of fish in which they occur, and the significant deviations of the transmissions from the control by the different types of cells. The arrangement of the species by families followed chiefly the classification of marine fishes given by Perlmutter (1961), supplemented by reference to classifications given by Smith (1898), Collins (1959), Herald (1961), and Hvass (1965).

Results and Discussion

Before going into a detailed examination of the significance of the results I wish to point out that an attempt has been made in Figure 1, to arrange the data so that the chief results of the investigation may be seen as a whole. Thus it is seen that there are cells from only one species in type A, that most of the cells in type B are in the Chondrichthyes, and most of the cells of type C are in the Osteichthyes. Except for one cell in the Marsipobranchii, all of the cells of type D are in the Osteichthyes. Not only are these distributions quite apparent by casual perusal of the chart, but the trends of the transmissions with reference to the control may be easily seen. With these facts in mind the data are now examined to see whether the method has possibilities for use as a taxonomic or experimental tool. In the following analysis of the data, the references to categories are kept as brief as possible. References to families are not entered in the chart, but are in parenthesis following the species name.

Marsipobranchii

There are no acidophile of types A or C in this class.

Type B. There is one measurement in *Petromyzon marinus* (Petromyzontidae), not significantly different from the control; and one from *Myxine glutinosus* (Myxinidae) with a significantly lower transmission.

Type D. One measurement from *Petromyzon*, significantly higher than the control. From these scant data there is a suggestion of significant differences in the acidophilic material of the two species probably caused by differences in density of the acidophilic material. There is however, no great diversity between the transmissions such as will be seen in these types of acidophils in the species of the other classes.

Chondrichthyes

Type A. Three measurements from *T. torpedo* (Torpedinidae), none of which is significantly different from the control.

Type B. Three measurements from *T. torpedo*, all significantly lower than the control. Two measurements from *Squalus acanthias* (Squalidae) both significantly lower than the control. Two measurements from *Raja erinacea* (Raidae), both significantly lower; four measurements from *R. laevis*, one not different, and three significantly lower; and two measurements from *R. radiata*, one not different, and one significantly lower than the control. Thus of the 13 measurements made only two (15%) are not different from the control; and eleven (85%) are significantly lower. There were none higher than the control. Hence, the data on these cells are regarded as giving evidence of a denser content and greater adsorption of the acidophilic dye than occurs in the acidophilic granules of the human eosinophils used as a control. The transmissions do not permit any evidence for familial or specific differences within the class, but they do indicate that the transmissions are significantly low and are fairly constant through-out the class.

Type C. Seven measurements from three species, from three families were made of this type. In five measurements from *Scyliorhinus stellatus* (Scyliorhinidae), all are signficantly higher than the control. In *Mustelus canis* (Triakidae) with one measurement, the transmission is significantly higher than the control. Also in *R. ocellata*, the one measurement is significantly higher. It is to be noted that in type C, there are no measurements significantly lower than the control. This is the reverse of the trend of the transmissions by type B and the difference is regarded as of value for use in discriminating between the species. One would expect the transmission to be lower in type C than B, because of the presence of basophilic matrix, but none of these measurements is lower than the control, all are higher. It would appear that this objective method shows up differences clearly in these Chondrichthyes.

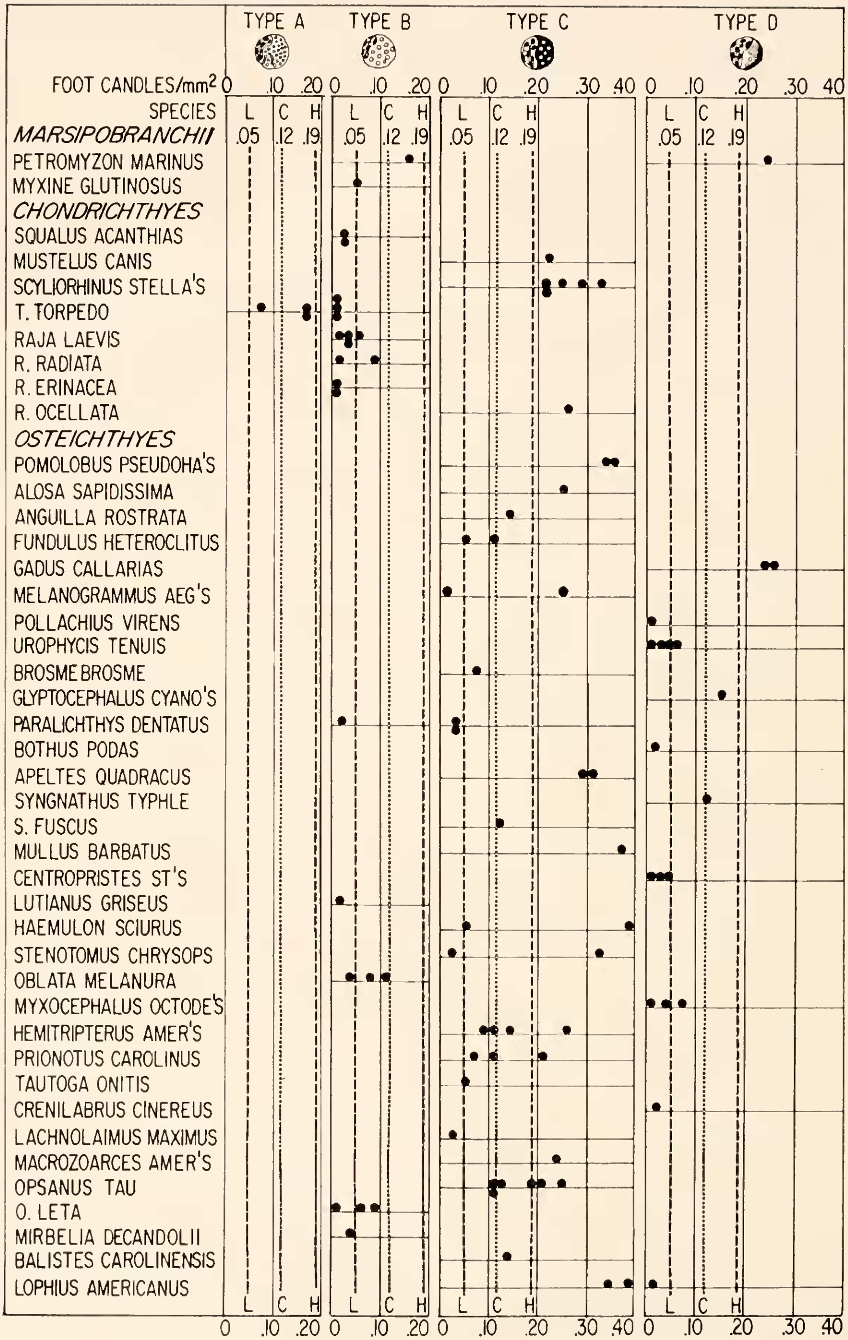

FIGURE 1. Acidophils of marine fishes. A chart showing the distributions of the transmission of monochromatic (red) light by the acidophilic areas of photomicrographs in foot

In looking over the transmissions by all type of cells in the Chondrichthyes, it can be seen that the transmission by cells of type A are not significantly different from the control, those of type B in which the largest number of species were studied and measurements made, have a greater number of transmissions significantly lower than the control; and in type C this trend is reversed.

Type D. There are no cells of type D present.

Osteichthyes

Type A. There are no cells of this type in the Osteichthyes.

Type B. There are only five species with acidophils of this type. When the distributions of the several measurements are examined, it is seen that in *Paralichthys dentatus* (Paralichthydae), the one measurement is significantly less than the control; of the three measurements in *Oblata melanura* (Sparidae) two are not different and one is lower than the control; in *Mirbelia decandolii* (Gobiesocidae), the one measurement is significantly lower; as is also the one measurement in *Lutianus griseus* (Lutianidae); and in three measurements from *Opsanus leta* (Batrachioididae), two are not significantly different from the control and one is lower. Of the nine measurements, four (45%) are not different from the control, and five (55%) are significantly lower, and none is higher. The cells of type B are not characteristic of the Osteichthyes and occur in the smallest number of species. Theoretically, because of the presence in most of the cells of a high percentage of clear area, the transmissions should be higher than lower.

Type C. This type of acidophil is characteristic of the Osteichthyes. It has been observed in twenty species from seventeen families, and is usually the only type of acidophil present in these species. Following down the list of measurements and their deviations from the control in Figure 1, it is seen that in *Pomolobus pseudoharengus* (Clupeidae) both measurements are significantly higher than the control; and in *Alosa sapidissima*, in the same family, the single measurement is also higher. In *Anguilla rostrata* (Anguillidae) the single measurement is not different from the control; in *Fundulus heteroclitus* (Poecillidae) both measurements are not different from the control; in *Melanogrammus aeglefinis* (Gadidae), one is higher and one is lower; and in *B. brosme* (Gadidae) the single measurement is not different; in *Paralichthys dentatus* (Paralichthydae) both measurements are significantly lower; in *Apeltes quadracus* (Gasterosteidae) both are significantly higher; in *Syngnathus fuscus* (Syngnathidae) the one measurement is not different from the control; in *Mullus barbatus* (Mullidae) the one measurement is significantly higher than the control; in *Haemulon sciurus* (Haemulidae), of the

candles/1.0 mm². At left, names of classes and species arranged in taxonomic order; four columns for transmissions by subjective types A, B, C and D arranged by foot candles/1.0 mm² from 0 to the highest transmission for each type; above each column is a diagram showing the distributions of the tinctorially visible areas in each type, acidophilic granules or vesicles (outlined white), clear areas (white not outlined) and basophilic areas, black. The dotted line at 0.12 ft-c (C) is location of control transmission for each type; dash lines at 0.05 (L) and 0.19 (H) ft-c are the lower and upper limits, respectively, of measurements not significantly different from the control as calculated by the X2 method for agreement; to the left of L, the measurements are significantly lower, and to the right of H, they are significantly higher than the control; solid circles, position of transmission for each measurement; horizontal lines, projection of the position of the species in relation to the type of cell.

two measurements, one is not different, and the other is significantly higher than the control; in *Stenotomus chrysops* (Sparidae), one is lower and one is higher; of the four measurements in *Hemitripterus americanus* (Hemitripteridae), three are not different and one is significantly higher than the control; of the three measurements in *Prionotus carolinus* (Triglidae), two are not different, and one is significantly higher. Of the two species of Labridae, the one measurement in *Tautoga onitis*, is not significantly different; and that from *Lachnolaimus maximus* is significantly lower than the control. The one measurement from *Macrozoarces americanus* (Zooarchidae) is significantly higher than the control. Of the six measurements from *Opsanus tau* (Batrachioididae) four are not significantly different from the control, and two are significantly higher. The single measurement from *Balistes carolinensis* (Balistidae) is not significantly different from the control. Of the two measurements from *Lophius americanus* (Lophidae) both are significantly higher than the control.

Reviewing and summarizing these distributions it is seen that of the 38 measurements made from twenty species having acidophils of type C, seventeen (45%) are not significantly different from the control; five (13%) are significantly lower, and sixteen (43%) are significantly higher. Examination of the deviations in the X2 columns, shows that there is not much deviation by the cells with low transmissions, while those with the higher transmissions have much greater deviations. When we localize these figures for species by using the transmission as a measure of classification, it is seen that there are six species having only acidophils not significantly different from the control; *Anguilla*, *Fundulus*, *Brosme*, *S. fuscus*, *Tautoga*, and *Balistes*. There are two species with only acidophils with transmissions significantly lower than the control, *Paralichthys* and *Lachnolaimus;* and six species with only acidophils with significantly higher transmission, *Pomolobus*, *Alosa*, *Apeltes*, *Mullus*, *Macrozoarces* and *Lophius*. Of the species in which variations of transmission occur, there are three species in which there are more cells with transmission not significantly different and with significantly higher transmission, (ratio in parenthesis); *Hemitripterus* (3:1), *Prionotus* (2:1), *Opsanus tau* (5:1); and finally, there are two species, *Melanogrammus* and *Stenotomus*, in which no non-significantly different cells occur and the ratio of significantly lower to significantly higher is equal.

Thus it would seem that in these cells of type C, there is a much greater tendency for the transmission not to be different from the control, a slight tendency for the cells to have a low transmission, and great tendency to deviate to a higher transmission. The deviation to higher and lower transmissions are suggested to be indications for significant modification of the acidophilic substance in these species, a modification which could not be detected without the use of the present method.

When the distributions of the cells of type B and their transmissions in the Osteichthyes are contrasted with type B of the Chondrichthyes, the most striking difference between them is the absence of cells with significantly higher transmission in the Chondrichthyes, and the tendency for most of the cells to have significantly lower transmissions than the control.

Type D. It should be recalled that the cytoplasm of the acidophils of this type contains a mixture of clear areas, and basophilic matrix, as a background for the acidophilic granules or vesicles. The areas occupied by these materials were taken into account in calculating the transmissions. There are ten species from eight families which have cells of this type. There is one family (Gadidae), in which three species were studied. Of the two measurements in *Gadus callarias* both are significantly higher than the control; the single measurement from *Pollachius virens* is significantly lower, while of the four measurements from *Urophycis tenuis*, two are not significantly different from the control; and two are significantly lower. In *Glyptocephalus cyanoglossus* (Pleuronectidae), the single measurement is not significantly different; in *Bothus podas* (Bothidae) the transmission is significantly lower; and in *S. typhle* (Syngnathidae) it is not significantly different; in *Centropristes striatus* (Serranidae), of the three measurements, all are significantly lower than the control; and in *Myxocephalus octodecimspinosus* (Cottidae) of the three measurements, two are not significantly different and one is significantly lower than the control; in *Crenilabrus cinereus* (Labridae) the single measurement is significantly lower than the control; as is the single measurement in *Lophius americanus*.

The distribution of these measurements in type D shows that the greatest number of the measurements are significantly lower than the control and the least number are significantly higher. These deviations are different from the trend toward higher measurements in type C.

It would seem that by this method the differences within the types can be used as a basis for objective classification of the species in which they occur. There are a few species in which cells of more than one type are present. In *Petromyzon* there are cells from types B and D; in *Torpedo* from A and B; in *Paralichthyes*, from B and C; and in *Lophius*, from types C and D.

Review of the distributions by class, type and transmission

Total number of species, 43. Distributions of the types by species in the several classes is as follows: (percentage of total number of species in parenthesis):

Marsipobranchii. Two species (4.7%); type A, none' type B one (2.3%), not different (2.3%); type C none; both type B and D, one (2.3%), not different from control and higher.

Chondrichthyes. Eight species (18.5%); type A and B in same species, one (2.3%); transmission lower and not different from control (2.3%); type B, four species (9.4%), two lower (4.7%); two not different and lower (4.7%); type C, three species (7.0%), all higher (4.7%), than the control; type D, none.

Osteichthyes. Thirty-three species (77%); type A, none; type B (only) four (9.4%), two not different and low (4.7%), two low (4.7%); type B and C in same species, one (2.3%), low (2.3%); type C alone, eighteen (42%); six not different from control (14.1%), three lower (6.2%), five higher (11.5%), three not different and higher (6.9%); type C and D in the same species one (2.3%), transmission lower and higher (2.3%); type D alone, nine species (21%); in two (4.7%), transmission not different from control, four (9.4%) lower than control, one (2.3%) higher, two (4.7%) transmission not different and lower in same species.

The distribution by type throughout the whole forty-three species is: type A only, none; type B only, ten (23%); type C only, twenty (46%); type D only, nine (21%); types A and B in one species, one (2.3%); B and C in one species, one (2.3%); B and D in one species, one (2.3%); and C and D in one species, one (2.3%).

Finally out of all the species in the different classes, the distribution of the types is: in the Marsipobranchii, two species, of which one (50%) has type B cells only; and the other has type B and D cells (50%); in the Chondrichthyes (eight species); types A and B in the same species, one (12.5%); type B only, four (50%); type C only, three (37.5%); and type D only, none. In the Osteichthyes, thirty-three species: type A, none; type B, four (12.1%); type C, eighteen (54.5%); type D, nine (27.2%); types B and C in the same species, one (3.2%); types C and D in the same species, one (3.2%). In the two classes in which there are more than three measurements, the incidence of types B and D are highest in the Chondrichthyes; and types C and D, in the Osteichthyes. The latter two types of acidophils differ from the others by the presence of basophilic matrix, which material may be taken as the characteristic substance of the acidophils of the Osteichthyes.

This investigation was undertaken as a pilot project to supplement the descriptions of the blood cells of fishes which I had made as part of a wider survey of the morphology of the blood cells of the Vertebrates (Kindred 1961). The blood cells had been photographed and described by the usual subjective methods, when in reviewing the material, I thought that an objective numerical value entered beside the subjective description, would be of great help in making comparisons between the characteristics of the blood cells of the different fishes. In this paper I have presented the result of such an attempt. I have obtained such objective data, but the question arises whether they can be used effectively in their present form, knowing that the preparation of the materials has been handicapped by the presence of some modifying factors; such as the method of preparation of the smears, thickness of the smears, amount of the plasma clinging to the smears, the chemical characteristics of the Wright's stain used, the degree of adsorption of the dyes and the uniformity of the photomicrographs. However, since it is known that these factors are not particularly taken into consideration at present in making morphological descriptions of the blood cells such as occur in the literature of the hematology of the leukocytes, it was decided to present my results as a preliminary pilot study which could be followed by a wider and more accurate evaluation of the problems involved in achieving the results. Thus there is a place in the methods of study of the blood cells under the present conditions which I hope I have partially filled by showing that objective results can be obtained, when the materials are prepared under the present methods, taking such precautions to influence uniformity as can be taken. The present objective results emphasize definite trends in the transmission of red light by certain tinctorial areas of the acidophils which may be utilized as objective differences between the acidophils of the different species of fish and which may be of taxonomic value in assessing differences between species or in changes in the acidophils brought about by experimentation.

The fishes furnished by the U. S. Bureau of Commercial Fisheries, Woods Hole, Massachusetts, were classified by Mr. Kenneth Cumming; fishes of Naples, Dr. P. Tardent of the Zoological Laboratory; and the few species from Florida, by Dr. Leonard J. Greenfield of the Marine Laboratory of the University of Miami.

SUMMARY

Highly magnified colored photomicrographs of the acidophilic granulocytes stained with Wright's stain of forty-three species of marine fishes were studied in an attempt to relate subjective descriptions of the cells to objective measurements of the transmission of monochromatic red light by the acidophilic materials (rods, granules, or vesicles). Four types of acidophils are classified by the distribution of the acidophilic materials, clear spaces and basophilic matrix of the cytoplasm. Objectively these types are classified statistically by the X2 method for deviations from a control transmission obtained in this case from the mean transmission by acidophilic materials in photomicrographs of human eosinophils prepared and photographed under the same conditions as was done in the fishes.

The data are presented in a chart (Fig. 1) showing the class, and species from which the cells came; type of cell (A, B, C, or D); transmission of the red light in foot candles/1.0 mm^2 by the acidophilic materials; range of significant deviations from the control as calculated by the X2 method.

Briefly, cells with only acidophilic granules, are found in only one species, *T. torpedo,* of the Chondrichthyes and the transmission is not significantly different from the control. Cells of type B, characterized by acidophilic materials (granules, rods or vesicles) and clear areas, predominate in the Chondrichthyes, and most of them have significantly low transmission; a few species of Osteichthyes, have such cells, and the transmissions are sufficiently variable to justify the use of the objective data as a tool in classification. Cells of type C, with acidophilic materials (granules and vesicles) and basophilic matrix, predominate in the Osteichthyes. There are a few species of Chondrichthyes which have them and in most of these cells the transmission is significantly higher than the control. In the Osteichthyes, these cells are characteristic and in practically all species, the transmission is either higher than the control, or not significantly different. Very few species have cells with significantly low transmission. Again it may be pointed out that the objective differences in the cells of type C permit a classification which could be of taxonomic value. Finally, the cells of type D, with acidophilic vesicles, clear spaces and basophilic matrix are not represented in the Chondrichthyes. One sample was found in the Marsipobranchii, but all the rest are in species of Osteichthyes. In only one species is the transmission significantly higher than the control, the transmissions by the cells of the other species are either not significantly different from the control, or are lower.

It is concluded from this pilot study that the objective results obtained from measurements of transmission of monochromatic red cell light by the acidophilic components in photomicrographs of the acidophils of fishes stained with Wright's stain, to warrant its use with modification as a tool in the objective study of blood cells in smears.

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